Growth response of Pterocarpus santalinus *seedlings to native microbial symbionts (arbuscular mycorrhizal fungi and* Rhizobium aegyptiacum*) under nursery conditions*

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ORIGINAL PAPER

Growth response of *Pterocarpus santalinus* seedlings to native microbial symbionts (arbuscular mycorrhizal fungi and *Rhizobium aegyptiacum*) under nursery conditions

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Abstract The objective of this research was to improve the growth and biomass of *Pterocarpus santalinus* L.f. (an endangered leguminous tree) using native microbial symbionts such as arbuscular mycorrhizal fungi and *Rhizobium* associated with native populations of *P. santalinus*. The native arbuscular mycorrhizal fungi isolated from *P. santalinus* soils were identified as (1) *Glomus fasciculatum*; (2) *Glomus geosporum*; and *Glomus aggregatum*. A nitrogen-fixing microbial symbiont was isolated from the root nodules of *P. santalinus* and identified as *Rhizobium aegyptiacum* by 16s rRNA gene sequencing. These microbial symbionts were inoculated individually and in combination into *P. santalinus* seedling roots. After 90 days, growth and biomass had improved compared with uninoculated controls. Shoot and root lengths, number of leaves, stem circumference, number of root nodules, biomass, nutrient uptake and seedling quality index were significantly increased by a combined inoculation of arbuscular mycorrhizal fungi + *Rhizobium aegyptiacum*. It was concluded that native microbial symbionts positively influenced *P. santalinus* seedling growth which will be helpful for successful field establishment.

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Keywords Arbuscular mycorrhizal fungi · Microbial symbionts · *Pterocarpus santalinus* · Red sanders · *Rhizobium aegyptiacum*

Introduction

Forest cover in India has been increased by 1% and total cover rose from 92,500 to 93,815 km^2 (ISFR 2017). This was primarily due to the establishment of different types of plantations, including commercial species such as casuarina and eucalyptus (Ravindranath et al. 2014). However, the southern part of India is now focused on planting native species rather than exotics in order to further increase tree cover, of which *Pterocarpus santalinus* L.f is one. *P. santalinus* is an endemic, leguminous species predominantly and naturally distributed in the tropical dry deciduous forests of Andhra Pradesh, India. This species, commonly called red sanders, has a moderate height with an erect bole and a dense, rounded crown. Although the natural distribution of this species is in India, Poudel (2003) reported outliers in the Tara Hill area of the Kaochi District of Nepal. The optimum altitude for *P. santalinus* is between 300 and 800 m a.s.l. and it is usually found on hilly slopes with sandy, loam soils (Raju and Nagaraju 1999).

Pterocarpus santalinus is slow-growing, attaining a harvestable size of 70 cm DBH at 80–100 years (UNEP-WCMC 2017). *P. santalinus* has potential medicinal as well as commercial values but it is an endangered species due to over exploitation for its medicinal properties and was categorized as endangered in 1998 by the International Union for the Conservation of Nature (UNEP-WCMC 2017). Global demand for *P. santalinus* was estimated at 3000 tonnes per year (Kukrety et al. 2013) and the value of wood per tonne was US $ 12,000 (Soundararajan et al.

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2016). A musical instrument, 'Shamisen', is made from *P. santalinus* wood in Japan (Arunkumar and Joshi 2014). The species yields a natural dye called 'santalin' used for neutraceuticals and in foods (Arunkumara et al. 2011). Chemicals of the heartwood are used for treating skin diseases, bone fractures, leprosy, spider poisoning and scorpion stings, for ulcers and mental aberrations (Arokiyaraj et al. 2008). Oral administration of the bark extract resulted in significantly reduced hypoglycemic activity in diabetic rats (Rao et al. 2001). The wood is considered astringent, antipyretic, anthelminitic (used to destroy parasitic worms) and diaphoretic. The wood is also in high demand for furniture, toys and handicrafts (Arunkumar and Joshi 2014). Bowls of the wood of this species have been used to hold drinking water as a treatment for diabetes (Nagaraju et al. 1991). The stem bark extract has an antibacterial property against numerous bacterial pathogens (Manjunatha 2006).

Pterocarpus santalinus is propagated by seeds and vegetative cuttings, although Prakash et al. (2006) were successful with micropropagation. The species is endangered due to illegal logging, poor natural regeneration and microclimate changes causing the degradation of natural populations (Sanjappa 2001), particularly in the Seshachalam forest area of Andhra Pradesh. *P. santalinus* is a nitrogen-fixing species and symbiotically associated with the N-fixing bacteria '*Rhizobium*'. These root nodule bacteria play vital roles in nitrogen fixation and secretion of growth substances helpful to plants for growth and biomass production, and therefore reduce the need for chemical nitrogen fertilizers (Herridge et al. 2008). The other microbial symbiont, arbuscular mycorrhizal (AM) fungi, is also associated with *P. santalinus* (Rajasekar et al. 2002) and help to improve phosphorus (P) and water uptake for improved growth (Muthukumar and Udaiyan 2018). However, there is little information available on AM fungi and *Rhizobium* application studies with *P. santalinus* (Rajasekar et al. 2002), and it is essential to identify the effects of these native microbial symbionts on the growth of in *P. santalinus* under nursery conditions. This could facilitate the production of quality seedlings for successful field establishment.

Materials and methods

Collection of root nodules and soil

Soil samples (100-g each) were collected from the rhizosphere beneath 50 *P. santalinus* trees in Seshachalam forest area, Tirupati, Andhra Pradesh (14° 33′ N, 78° 25′ E; 853 m a.s.l.). Mean annual rainfall of the area 181 mm (±2.8) and mean annual temperature is 31.1 °C (±1.3). This dry deciduous forest area is dominated by the natural populations of *P. santalinus* along with *Cycas beddomei* Dyer, *Terminalia pallida* Brandis, *T. chebula*, *Syzgium alternifolium*, *Shorea tambaggia* and *Boswellia ovalifoliolata*. Root nodules of *P. santalinus* were also collected from the natural populations of *P. santalinus* and and stored at −4 °C. Physical and chemical properties of the soil determined according to Jackson (1973). The pH was 5.9 (±0.2), electrical conductivity 0.19 (±0.01), nitrogen (N) 20.2 (±0.5) mg kg^{-1}, phosphorus 14.2 (±1.1) mg kg^{-1} and potassium (K) 31.5 (±1.3) mg kg^{-1}.

Isolation and culture of arbuscular mycorrhizal (AM) fungi

AM fungal spores were isolated from the soil samples according to Gerdemann and Nicolson (1963). The samples contained 5.2 (±0.3) infective propagules, (spores, hypha), as analyzed by the most probable number method (Porter 1979). The isolated spores were identified by the original description in the manual of Schenck and Perez (1990) and were identified as: (1) *Glomus fasciculatum* Gerd. & Trappe emend. Walker & Koske (Fig. 1a); (2) *Glomus geosporum* (Nicol & Gerd.) Gerd & Trappe (Fig. 1b); and, *Glomus aggregatum* N.C. Schenck & G.S. Sm (Fig. 1c). These AM fungi were multiplied on sterile medium (alfisoil and sand) with *Zea mays* L. under

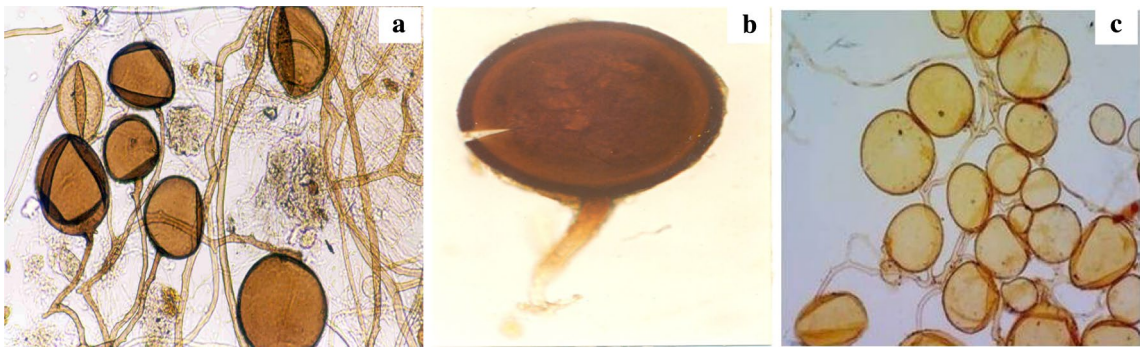

Fig. 1 **a** *Glomus fasciculatum*; **b** *Glomus geosporum*; **c** *Glomus aggregatum*

greenhouse conditions, (65% average relative humidity and 22 °C average temperature) for 4 months in pot cultures.

Isolation and culture of *Rhizobium*

Root nodules from *P. santalinus* roots were surface sterilized with 30% hydrogen peroxide (H_2O_2) at room temperature for 10–20 min. Under aseptic conditions, the nodules were rinsed in sterile water and 0.3 g of root nodule was ground manually in a sterile mortar and pestle. The nodule solution was centrifuged at 1000 rpm for 20 min and the supernatant filtered through Whatman No.1 filter paper. The suspension was then spread on Yeast Extract Mannitol Agar Medium (YEMA) (Graham 1969) plates and incubated at 25 °C for 15 days. One litre of YEMA contains 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 10 g mannitol, 1 g yeast extract and 20 g agar. In addition, a 1% Congo red solution was added to the medium. The pH was 6.8. After 15 days of incubation, *Rhizobium* growth appeared as slippery white colonies (Fig. 2) which were then into a YEMA broth for up-scaling the inoculum. The broth cultures were incubated at 32 °C in 250-mL flasks in an orbitol shaker (Gyromax 703R, Concord, CA, USA) at 1000 rpm for 20 min. The cultures were then homogenized at 1000 rpm for 10 min and stored at 4 °C. The culture was characterized at a molecular level after DNA extraction by polymerase chain reaction and sequenced through 16s rRNA using the *Rhizobium* specific primers recAf and recAr (Bournaud et al. 2017). Phylogenetic analysis of the nucleotide was performed with MEGA6 and identified as *Rhizobium aegyptiacum* and deposited in the National Center for Biotechnology Information in the United States as accession No MH665677 (Karthikeyan and Arunprasad 2018).

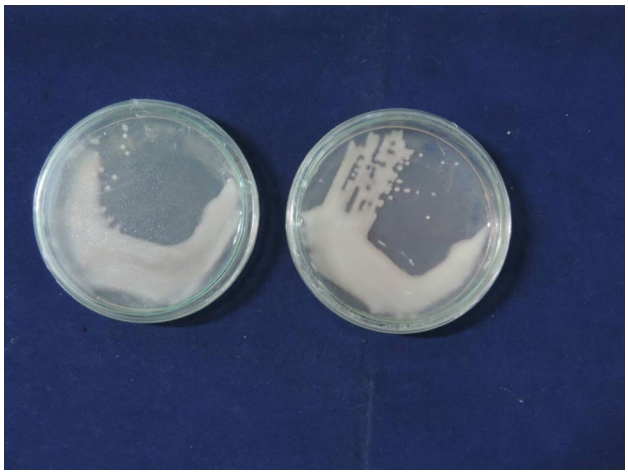

Fig. 2 *Rhizobium* colonies

Propagation of *P. santalinus* seedlings

Seeds were immersed in cold water for 48 h and sown in sterile sand. After 25 days, the seedlings had germinated and ones with similar heights (7 cm) were transplanted to polythene bags (14 × 27 cm) containing 3 kg of steam-sterilized (100 °C for 3 h at 103.5×10^3 Pa) alfi soil + sand (1:1), kept in a shade house and watered twice per day.

Inoculation with AM fungi and *Rhizobium aegyptiacum*

Pot cultures of *Z. mays* containing 20 g of chlamydospores of the three AM fungi were placed 5-cm below the soil surface in each polythene bag. Inoculation of cultured *Rhizobium aegyptiacum* was achieved by applying 10 ml of rhizoidal suspension in the root zone in each seedling bag of the rhizoidal treatments. These microbial symbionts were inoculated individually and in combinations. An uninoculated control treatment was also maintained. Therefore, there were four treatments, namely, (1) control, (2) AM fungi (20 g), (3) *Rhizobium aegyptiacum* (10 mL), and (4) AM fungi (20 g) + *Rhizobium aegyptiacum* (10 mL) with 15 replicates of each treatment containing five seedlings per treatment, for a total of 300 seedlings set out in a randomized block design. The inoculated and uninoculated treatments were maintained for 90 days under shade house conditions at 26.2 °C and 65.4% relative humidity. The seedlings were watered regularly to maintain turgidity.

Harvest and analyses

Ninety days after inoculation, the seedlings were harvested with intact root systems. Root and shoot lengths, stem circumference, number of root nodules, root biomass and number of leaves were measured. Shoot and root biomass were determined after oven drying at 50 °C for 48 h.

AM fungal colonization

A portion of the harvested root samples was processed for microscopic observation according to Philips and Hayman (1970), and per cent root inoculation was assessed by the root slide method of Read et al. (1976).

Seedling quality index

This was calculated using the formula of Dickson et al. (1960), namely:

$$SQI = S^1DW / [SS/RCD + S^2DW/RDW]$$

where SQI means seedling quality index, SDW seedling dry weight (g), S^1S seedling height (cm), RCD root collar diameter (mm), S^2DW shoot dry weight (g), RDW root dry weight (g).

Tissue nutrient analysis

Dried (5-g) root and shoot samples were digested with a catalyst mixture (potassium sulphate and copper sulphate; 5:1) and triple acid (nitric acid + sulphuric acid + perchloric acid; 9:3:1) in a Kjeltec digestion system at 420 °C for 1 h. The samples were analyzed for N, P, and K levels according to Jackson (1973).

Statistical analysis

Data were statistically analyzed using Duncan's multiple range test in Statistical Package for the Social Sciences, USA Inc. ver. 16.

Results

Growth and biomass

The microbial symbiont-inoculated seedlings showed significantly improved growth and biomass ($p < 0.05$) compared to control seedlings (Table 1). *R. aegyptiacum*-inoculated seedlings had significantly higher shoot and root lengths and biomass than AM fungi-inoculated seedlings. However, the combination of *R. aegyptiacum* with AM fungi-inoculated seedlings had significantly higher growth (59.9 cm) and biomass (44.4 g plant^{-1}) than inoculations of microbial symbionts alone. There was a significant ($p < 0.05$) increased in the number of leaves (17.8 plant^{-1}) and number of nodules (20.6 plant^{-1}) with the AM fungi + *R. aegyptiacum* treatment (Table 1). Nodule biomass was also significantly increased in the AM fungi + *R. aegyptiacum* (0.00035 g) treatment. A lower root to shoot ratio (0.44) was significant ($p < 0.05$) in the combined microbial symbiont-inoculated seedlings compared with individual microbial treatments (Table 1; Fig. 3).

Fig. 3 Growth responses of *P. santalinus* seedlings inoculated with AM fungi and *Rhizobium aegyptiacum* (T1; AM fungi; TII; *Rhizobium aegyptiacum*; TIII; AM fungi + *Rhizobium aegyptiacum*)

AM fungal colonization

A higher percent of inoculation was observed in AM fungi-inoculated seedlings (95.6%) which is similar to AM fungi + *R. aegyptiacum* (94.3%) (Figs. 4, 5).

Seedling quality index

This was significantly high ($p < 0.05$) in the seedlings inoculated with both AM fungi and *R. aegyptiacum.* A significantly low quality index was recorded for the control seedlings (Fig. 6).

Table 1 Growth and biomass of *P. santalinus* inoculated with AM Fungi and/or *Rhizobium aegyptiacum* (mean of 15 replicates)

Treatments	Shoot length (cm)	Root length (cm)	No. of leaves plant^{-1}	No. of nodules plant^{-1}	Stem girth (mm)	Root biomass (g)	Shoot biomass (g)	Nodule biomass (g plant^{-1})	R/S ratio
Control	6.5a	14.4a	3.6a	–	0.85a	2.8a	3.3a	–	0.83b
AM fungi	12.8b	28.3c	10.5c	–	1.5b	6.8b	10.4b	–	0.65a
Rhizobium aegyptiacum.	20.5c	22.4b	6.4b	15.8a	1.8ab	12.2c	25.3c	0.00029a	0.48c
AM fungi + *Rhizobium aegyptiacum*	28.3d	31.6d	17.8d	20.6b	2.0c	13.6c	30.8d	0.00035b	0.44d

Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test ($p < 0.05$)

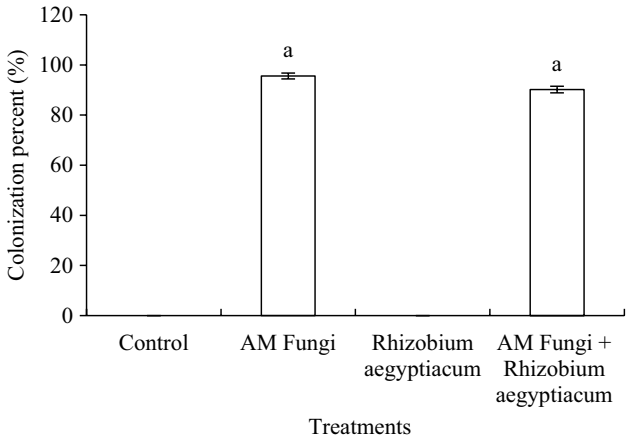

Fig. 4 AM fungal colonization in *P. santalinus* seedlings. Bars indicating same letters are not significantly different according DMRT ($p < 0.05$). Error bar indicating SE (±) of mean

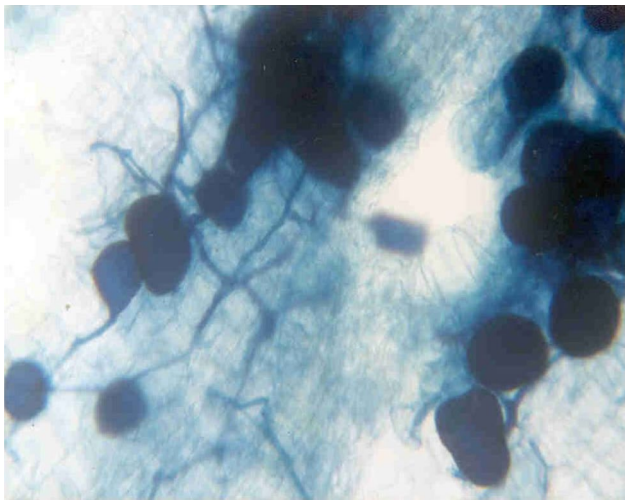

Fig. 5 AM fungal infection showing vesicles in *P. santalinus* seedlings

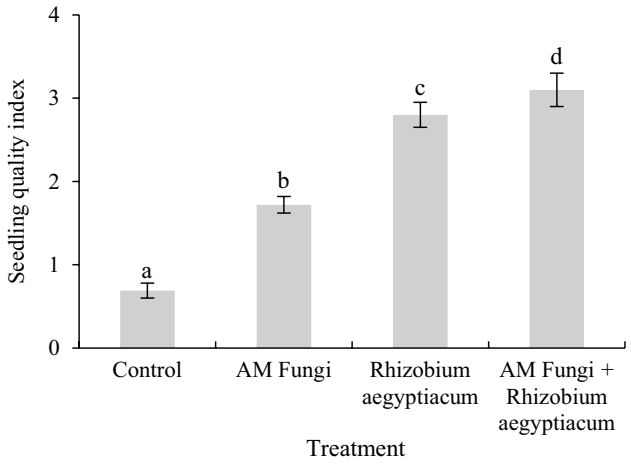

Fig. 6 Seedling quality index of *P. santalinus* seedlings with inoculation of AM fungi and *Rhizobium aegyptiacum* (mean of 15 replicates). Bars indicating same letters are not significantly different according DMRT ($p < 0.05$). Error bar indicating SE (±) of mean

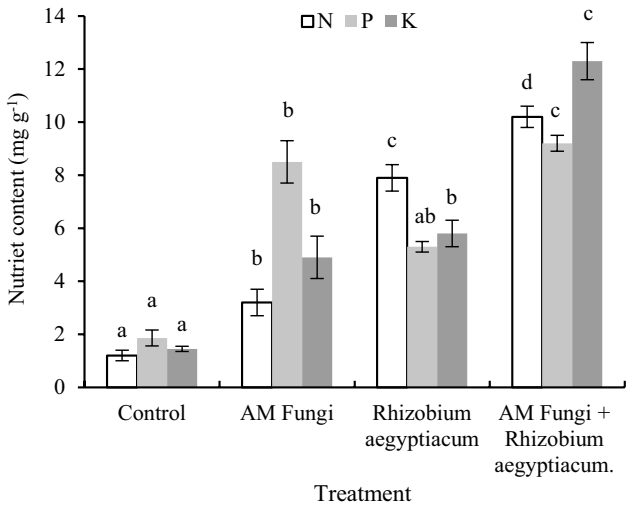

Fig. 7 Nutrient content of *P. santalinus* seedlings inoculated with microbial symbionts (mean of 15 replicates). Bars indicating same letters are not significantly different according DMRT ($p < 0.05$). Error bar indicating SE (±) of mean

Tissue nutrient contents

N, P and K levels had significantly (increased ($p < 0.05$) in seedlings inoculated with the combined microbial symbionts compared to the controls and the individually inoculated microbial symbionts (Fig. 7).

Discussion

The results of this study show that improved growth of *P. santalinus* is directly related to the microbial symbionts (AM fungi and *R. aegyptiacum*). Inoculation of *P. santalinus* seedlings with native *R. aegyptiacum* or AM fungi (*Glomus fasciculatum*, *Glomus geosporum* and *Glomus aggregatum*), either individually or in combination increased growth and biomass compared with the controls. Arbuscular mycorrhizal fungi can improve growth by increasing the uptake of nutrients from soils (Gerdemann 1975). As a result, *P. santalinus* thrives even in low nutrient soils along with the other nitrogen-fixing microbial symbiont (Khan et al. 2014). However, comprehensive studies between leguminous tree species and microbial interactions are limited (Muthukumar and Udaiyan 2010). In this study, *P. santalinus* seedlings showed improved quality in height and biomass because of

inoculation with microbial symbionts, which required for early and successful field establishment. Prasad et al. (2012) also reported that arbuscular mycorrhizal fungi increase root length. Similarly, the number of leaves of *P. santalinus* increased in inoculated seedlings, especially due to the effect of AM fungi. In previous studies, leaf numbers of *Tectona grandis* L.f. (Rajan et al. 2000) and *Macadamia tetraphylla* (Yooyongwech et al. 2013) increased due to the inoculation with AM fungi. This occurred not only in leguminous trees but also in legume crops such as the common bean, *Phaseolous vulgaris* L. (Korir et al. 2017) and pea, *Pisum sativum* L. (Bai et al. 2016). This synergistic effect by arbuscular mycorrhizal fungi and other growth-promoting bacteria, including *Rhizobium*, has been reported.

Rhizobium aegyptiacum and arbuscular mycorrhizal fungi had significantly positive effects growth and nutrient uptake by *P. santalinus* seedlings. Similar results have been reported by Lammel et al. (2015) for *Mimosa* spp. Increased numbers of nodules by inoculation with indigenous strains of *R. aegyptiacum* and arbuscular mycorrhizal fungi in *P. santalinus* were found in this study. Diouf et al. (2005) also reported similar results for *Acacia auriculiformis* and *A. mangium*. Inoculation of both AM fungi and *R. aegyptiacum* increased nodule biomass in *P. santalinus.* This is in agreement with Karthikeyan and Muthukumar (2006), and may be the reason for the need for sufficient energy in the form of adenosine triphosphate to produce root nodules by *R. aegyptiacum* and that energy is supplied by arbuscular mycorrhizal fungi (Muthukumar and Udaiyan 2018). Large amounts of nodular tissue concentrations of leghemoglobin and acetylene were reported in arbuscular mycorrhizal fungi + *Rhizobium*-associated legume plants (Daft and El-Giahmi 1975). For these reasons, legume species like *P. santalinus* prefer more than one root symbiont under natural conditions. *P. santalinus* seedlings inoculated with arbuscular mycorrhizal fungi + *R. aegyptiacum* resulted in decreased root to shoot ratios by increasing above ground production and possibly by reducing the need for below ground production since the function of nutrient acquisition was taken over by AM fungi (Smith 1980; Smith and Smith 2012). Muthukumar and Udaiyan (2018) also reported reduced root to shoot ratios in *Acacia auriculiformis* with inoculations of arbuscular mycorrhizal fungi and other growth promoting rhizobacteria.

The higher percentage of arbuscular mycorrhizal fungal colonization in *P. santalinus* suggests that these native fungi may have more infective propagules that colonize the roots (Muthukumar and Udaiyan 2018). Increased N, P, and K in the inoculated seedlings may be due to several factors, including the effects of the tripartite symbiotic association (legume + *R. aegyptiacum* + AM fungi), attributed to the combination of arbuscular mycorrhizal fungi and *R. aegyptiacum* as significantly increasing N contents, and AM fungi increasing P contents (Redante and Reeves 1981; Smith and Smith 2012; Muthukumar and Udaiyan 2018). Increased levels of K in *s*eedlings inoculated with microbial symbionts showed that potassium may act as an enzyme activator and may also stimulate nodule activity (Karthikeyan and Muthukumar 2006). Therefore, the microbial symbionts mobilized available nutrients to *P. santalinus* seedlings under nursery conditions and helped increase the uptake of nutrients (Chen et al. 2008). These results also have been confirmed with *Brugeira sexangula* (Lour.) Poir. (Karthikeyan and Sivapriya 2018), *Casuarina equisetifolia* (Karthikeyan 2017), *Mimosa scabrella* and *M. bimucronata* (Lammel et al. 2015). Further, the increased seedling quality index of inoculated *P. santalinus* seedlings highlights the benefits to growth improvement and nutrient uptake from inoculation. This supports earlier studies with forest tree seedlings (Karthikeyan and Muthukumar 2006; Muthukumar and Udaiyan 2010).

Conclusion

Indigenous microbial symbionts, arbuscular mycorrhizal fungi (*Glomus fasciculatum*, *Glomus geosporum* and *Glomus aggregatum*) and N_2-fixing bacteria (*Rhizobium aegyptiacum*) increased the growth and biomass of *P. santalinus* seedlings by providing essential levels of phosphorous nitrogen under nursery conditions. Therefore, these microbial symbionts should be used to improve *P. santalinus* seedling quality to help conserve this endangered species. Further, to avoid the deleterious effects of using chemical fertilizers in the nursery, the use of microbial symbionts that fix atmospheric nitrogen and/or increase nutrient uptake will be an environmentally benign approach for nutrient management of *P. santalinus* seedlings.

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